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Grant No. N00014-90-J-1648
Principal Investigator: Thomas G. Burke, Ph.D.
March 15, 1991: Report No. 1

City of Hope National Medical Ctr
Duarte, CA

PERFORMANCE REPORT

During the last four months of our project entitled "Evaluation of Liposome-Encapsulated Hemoglobin/LR16 Formulation as a Potential Blood Substitute", efforts have been made in the following four areas: 1) the resynthesis of LR16; 2) physical characterization of LEH/LR16 formulations; 3) design and development of membrane-impermeable LR16 analogues; and 4) preparations for small animal testing of surrogate blood preparations. The work completed in each of these areas is outlined below.

Resynthesis of LR16

Through the generosity of Dr. I. Lalezari of the Organic Chemistry Laboratory of the Montefiore Hospital and Medical Center, Bronx, NY, a 200 mg batch of LR16 was produced for our studies. This material was received March 12, 1991; we are presently evaluating the purity of this new batch of LR16 using the analytical techniques of high performance liquid chromatography and mass spectrometry. We are also presently in the process of evaluating the biological activity of the new LR16 material in purified human hemoglobin suspensions.

In our previous performance statement, we reported that Dr. Alok Singh of the Naval Research Laboratory had prepared a batch of LR16. However, this material was found to contain significant impurities and thus it will not be utilized in our work.

Physical Characterization of LEH/LR16 Formulation

As previously reported, we have been employing spectroscopic and microscopic methodologies in order to characterize our LEH material. Our preliminary findings concerning the LEH/LR16 material were reported in a paper entitled: "Liposome-Encapsulated Hemoglobin: Use of LR16 Analogues in the Optimization of Its Oxygen Binding Properties", presented on 1/25 at the 35th Annual Meeting of the Biophysical Society, held in San Francisco, CA (see attached abstract).

In other recent studies, we have evaluated the ability of LR16 to diffuse out of the liposomes of interest. Figure 1 shows the oxygen dissociation curves taken on various LEH suspensions in 100 mM Hepes with 0.9% NaCl(pH 7.4). Shown in Figure 1 are data for: 1) a control sample of LEH (P_{50} value of 15.5 mm Hg); 2) a sample of LEH in which 0.5 mM LR16 has been coencapsulated (P_{50} = 27.5 mm Hg); and 3) the same LEH/LR16 suspension but after it had been washed twice with phosphate buffered saline (P_{50} value of

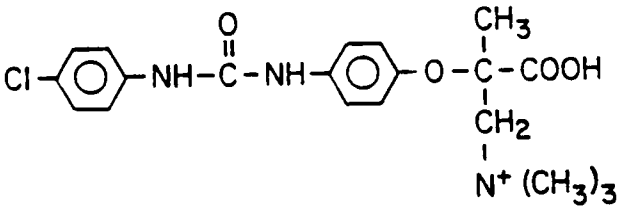
16.5 mm Hg). The LEH/LR16 was washed in PBS buffer and then centrifuged at 16,000 rpm.

Our results demonstrate that the LR16 drug is capable of diffusing out of the hemosomes. This unwanted drug diffusion is why we are so interested in developing membrane-impermeable LR16 analogues. Our efforts concerning analogue development are outlined in the following section.

Design and Development of Membrane-Impermeable LR16 Analogues

Because LR16 is capable of diffusing out of liposomes, we are interested in developing membrane-impermeable analogues. It is becoming increasingly more apparent through our studies that the synthetic development of effective, membrane-impermeable analogues will be a key objective in achieving our longterm goal of a liposome-encapsulated hemoglobin (LEH) substitute with optimal oxygen binding properties. In order to best accommodate this demand for new drug analogues, I have enlisted the assistance of Dr. Waldemar Priebe, Ph.D., a highly-regarded medicinal chemist on the faculty at The University of Texas M.D. Anderson Hospital, to the project (see attached letter).

We have presently identified a post-doctoral fellow (Stanislaw Ostrowski, Ph.D.) who will begin the synthetic development of membrane-impermeable analogues. We anticipate his hiring to the project to be complete by April 15, with the initiation of the synthetic research to begin soon thereafter. The first analogue which we intend to develop contains a permanently-charged (and thus membrane-impermeable) N,N,N-trimethylamino functionality (see structure below). Our interest in an analogue containing a N,N,N-trimethylamino functionality stems from the fact that permanently-charged molecules are membrane-impermeable because they cannot achieve an electroneutral form which is necessary for the diffusion of the compound through the acyl chain region of low dielectric constant. Upon completion of the synthesis, it will be of interest to evaluate the level of biological activity exhibited by the new LR16 analogue.



Structure 1. N,N,N-trimethylamino congener of LR16.

Preparation for Small Animal Testing of Surrogate Blood Preparations

As outlined in our grant proposal, we will test the efficacy of LEH/LR16 formulation in small animals during the second and third years of funding. Prior to our conducting these types of experiments, approval from the Research Animal Care Committee (RACC) of the City of Hope was required. This approval has recently been granted (see attached).

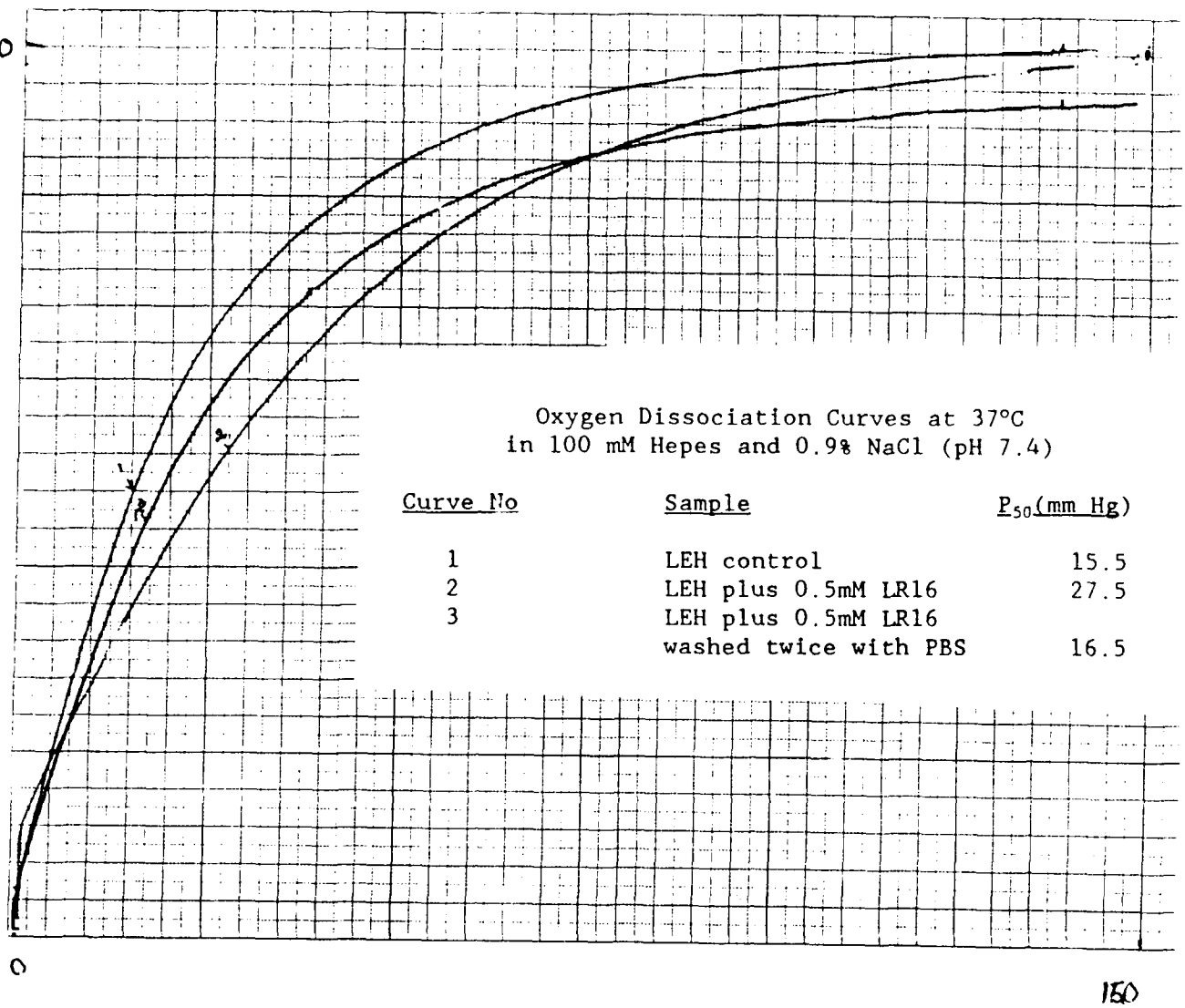


Figure 1. Oxygen dissociation curves which demonstrate that the LR16 drug can diffuse out of hemosomes. The LEH/LR16 vesicles were washed twice in PBS, resulting in a reduction in the observed P_{50} value (curve 3).

M-Pos471

ELECTROPORATION BY BIPOLAR ALTERNATING ELECTRIC FIELDS

Tian Y. Tsong, Piotr Marszalek, Jan Gimsa & T.-D. Xie. Department of Biochemistry, University of Minnesota, St. Paul, MN 55108

Electroporation by alternating electric fields (ac) has been shown to be gentler than direct current (dc) electroporation to the cells. A transfection efficiency of 5.2×10^{3} /μg DNA was obtained for E. coli (JM105) with plasmid PUC 18 DNA when the cell/DNA mixture was exposed to a 200 V/cm ac field of 30 s duration. This was nearly 10^{3} fold higher than the transfection efficiency of cells which were not exposed to an ac field, although cell survival was identical in both samples. Data also showed that the transfection efficiency was strongly dependent on the frequency of the applied field. The frequency dependence of membrane electroporation was examined by the entry of a fluorescence probe, propidium iodide, into murine myeloma cell line (Tib9). Propidium iodide is weakly fluorescent but becomes strongly fluorescent when bound to DNA. When an ac field reached a critical strength for membrane poration, the dye permeated into the cell. Within a few seconds, two narrow fluorescence bands appeared on the two loci facing the electrodes. The dye diffused into and completely permeated the cells within 2 - 5 min. The critical field strength for membrane poration was found to depend on the ac frequency as described by the Schwan Equation, $\Delta\psi_{crit} = 1.5\, R_{cell}\, E^{\circ}_{crit} / [\,1 + (2\pi f\tau)^{N}\,]^{2}$, in which $\Delta\psi_{crit}$, R_{cell}, E°_{crit}, f, and τ are the critical membrane breakdown potential, the radius of the cell, the critical amplitude of the applied field, the frequency and the membrane relaxation time, respectively. Electroporation under a rotating ac field was also performed to demonstrate that cell manipulation to produce uniform membrane poration could be achieved under controlled conditions.

[Supported by a grant from Office of Naval Research.]

M-Pos472

SURFACE SPECIFIC RECOGNITION OF FLUORESCENT CONJUGATED STREPTAVIDIN-PHYCOERYTHRIN PROTEINS ONTO BIOTIN LIPID LB MONOLAYER FILMS. L. Samuelson*, P. Miller+, D. Galotti, K.A. Marx, J. Kumar+, S. Tripathy, and D. Kaplan*, *Biotechnology Branch, US Army Natick Labs, Natick, MA 01760; Depts. of Chemistry and +Physics, University of Lowell, Lowell, MA 01854.

The Langmuir-Blodgett technique has been used to simultaneously orient and couple the photodynamic, water soluble protein, phycoerythrin, to a biotin derivatized phospholipid monolayer film. It was found that both avidin and streptavidin phycoerythrin conjugates will preferentially adsorb to the biotinylated lipid monolayer films while at the air-water interface. Pressure-area isotherms indicate that oriented monolayer films are formed with the hydrophilic biotin containing head groups exposed to the four biotin binding sites ($K_a = 10^{15}$) on avidin and streptavidin in the conjugated proteins. The binding of protein to the lipid films was determined by probing the characteristic, intense fluorescence of the phycoerythrin at 576 nm. The measurements were carried out on monolayer films transferred onto solid glass supports, exciting the samples with 496 nm light and scanning the emission from 515 to 670 nm. It was determined that avidin conjugated proteins complex to the biotin lipid monolayer through both specific and non-specific binding mechanisms, while the streptavidin-phycoerythrin binds by what appears to be only a specific (biotin-streptavidin) mechanism. Studies involving the mixture of these biotinylated phospholipids with a conducting polymerized surfactant system have been initiated in an attempt to enhance the stability of the films and elicit novel electronic and optical properties for potential bio-optoelectronic and structural research applications. The results will be presented.

M-Pos473

SOLUBILITY OF GASES IN BLOOD PLASMA AT HIGH PRESSURE. Gary T. Holm, Richard P. Kennan, and Gerald L. Pollack, Department of Physics and Astronomy, Michigan State Univ., East Lansing, MI 48824.

Most of the gas mixture breathed by divers under pressure is inert gas. The solubility and diffusion of inert gases in body fluids are therefore important for understanding decompression sickness. In this paper we discuss measurements of the solubility in blood plasma of three nonreactive breathing gases: helium, nitrogen, and hydrogen. The measurements were carried out in the temperature range 10-37°C at a pressure of about 30 atmospheres, corresponding to a depth of about 300 meters of water. For He, the Ostwald solubility L in blood plasma is about 5-7% depending on the temperature, smaller than it is in distilled water. The smaller solubility may be a salting-out effect due to the dominant sodium cation in blood plasma. At 25°C we obtained for He the results: L(in *plasma*)=0.0089 and L(*in water*)=0.0095. We also report related measurements on the solubility of xenon, in the form of the radioactive isotope Xe-133, at low pressures in blood plasma, blood substitute, and mixtures of blood plasma with blood substitute. The results of the experiments will be analyzed using standard thermodynamic and statistical mechanical methods. Entropies, enthalpies, and Gibbs energies of solubility will be discussed in terms of molecular interactions and compared with other systems.

Supported by ONR-NMRDC Grant No. N00014-88-K-0287.

M-Pos474

LIPOSOME-ENCAPSULATED HEMOGLOBIN: USE OF LR16 ANALOGUES IN THE OPTIMIZATION OF ITS OXYGEN BINDING PROPERTIES. Thomas G. Burke, Yayesh Asmerom*, Alok Singh*, and Sam Rahbar*, Dept. of Medical Oncology and Therapeutics Research, and *Department of Hematology and Bone Marrow Transplantation, City of Hope National Medical Center, Duarte, CA 91010 and *Center for Bio/Molecular Science and Engineering, Naval Research Laboratory, Washington, DC 20375

Reducing the oxygen affinity of liposome-encapsulated hemoglobin (LEH) to a physiologically-optimal range is a key step in the development of an LEH product that may potentially be used as a blood replacement fluid. We have shown that the phenylureido-substituted phenoxyisobutyric acid compound referred to as LR16 [Galezari et al (1988) PNAS 85, 617] is effective at modulating the oxygen affinity of purified human hemoglobin microencapsulated in lipid vesicles. In these experiments, hemoglobin was purified from outdated human blood and concentrated to 2.75 mM using pressure ultrafiltration. Hemoglobin solution (1 ml) and LR16 together were encapsulated in synthetic membrane materials (18 mg) consisting of DMPC, DPPC, DSPC, cholesterol and DMPG (ratios of 1:1:1:2:0.3, respectively). Liposomes of relatively uniform size (mean diameter between 2-3 um as determined by freeze fracture electron microscopy) were formed by successive extrusions through polycarbonate membrane filters of decreasing pore size. Whereas the P_{50} value for LEH in the absence of drug was 9 mm Hg, the inclusion of LR16 at concentrations of 0.1 mM, 0.2 mM, 0.5 mM, 1 mM, and 1.25 mM resulted in higher P_{50} values of 10 mm Hg, 13 mm Hg, 19 mm Hg, 27 mm Hg and 30 mm Hg, respectively. Thus, the presence of LR16 allows the oxygen dissociation curve of LEH to be right-shifted to a physiologically more relevant P_{50} range. Such a pharmacological approach provides a means of titrating systematically the P_{50} values of LEH formulations. This work was supported by Office of Naval Research Grant No. N00014-90-J-1648 to TGB.

MD ANDERSON
CANCER CENTER

January 22, 1991

Thomas G. Burke, Ph.D.
City of Hope National Medical Center
1500 E. Duarte Road
Duarte, CA 91010-0269

Dear Dr. Burke:

This is to indicate this Institution's willingness to participate with the City of Hope National Medical Center in a research project, entitled "Novel Allosteric Effectors of Human Hemoglobin". That portion of the project to be conducted at this Institution will be under the direction of Dr. Waldemar Priebe, Department of Medical Oncology. It is our understanding that the primary performance site will be at the City of Hope National Medical Center and that you will be the Principal Investigator. The enclosed budget outlines our cost estimates for a period of two years.

We shall be pleased to provide any additional information you may require.

Sincerely yours,

Frederick F. Becker, M.D.
Vice President for Research

FFB:jw

Enclosures

cc: Comptroller, UT System
Dr. Waldemar Priebe

NOTICE OF RESEARCH ANIMAL CARE COMMITTEE (RACC) ACTION

TO: Dr. Thomas Burke - Medical Oncology

FROM: William J. Burinda, RACC Secretary
Research Administrator

DATE: February 14, 1991

RACC #: 90000-91-01-2

PROTOCOL ENTITLED: "EVALUATION OF LIPOSOME-ENCAPSULATED HEMOGLOBIN/LR16 FORMULATION AS A POTENTIAL BLOOD SUBSTITUTE"

At its meeting on February 5, 1991, the RACC reviewed the above named protocol and took the following action:

APPROVED UNTIL 1/9/92

Note: **During the 12 month period covered by the RACC approval, you must advise the Research Animal Care Committee of <u>any</u> changes in the protocol or unexpected problems involving animals, as soon as you are aware of them.**

Signed: _____ Date: 2/15/91
William J. Burinda, RACC Secretary
Research Administrator

<u>NOTE: Please sign and return to RACC, c/o Research Administration.</u>

As an investigator of the City of Hope/Beckman Research Institute, I accept the responsibility to see that the approved protocol and RACC modifications to that protocol along with all Federal, State and Institutional laws, rules or guidelines relative to the care and use of animals will be conformed to in carrying out this research protocol, #90000-91-01-2

Signed: Thomas D. Burke
Investigator

Date: February 19, 1991

WJB/smb
Revised 6/13/90